

IDENTIFICATION AND GENOTYPING OF HEPATITIS B VIRUS BY PCR ASSAY USING GENOTYPE SPECIFIC PRIMERS

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Abstract

Eight genotypes of hepatitis B virus are recognized which show deference in geographical distribution. Differences in genotypes affect the severity, complication, course and treatment of disease. This study was established in Sulaimani city, Kurdistan region, Iraq to detect HBV genotypes by rapid and specific method using PCR, which based on type specific primers.

A total of 19 suspected hepatitis B patients including 14 males (73.68%) and 5 females (26.32%) were examined by PCR assay with universal outer primer used for detection of all HBV genotypes. The positive samples then examined by PCR assay using specific primers for each genotype.

The result showed that 15 cases were negative and 4 cases were positives for HBV. The 4 positive HBV samples were subjected to genotyping. 4 genotypes (A, B, C, D) out of 6 genotypes, which can be determined by this method, were identified. Unique results were detected. Firstly, all of the samples showed mix infection, in which 1 case was mix genotype B+C+D and 3 cases were mix genotypes of A+B+C+ D. Secondly, sequencing of D genotype showed 120 nucleotide deletion mutations which encode overlapping gene of polymerase and pre-S protein. These results were not or rarely reported

Keywords: Hepatitis B, PCR, genotyping, Sulaimani

Introduction

Hepatitis B infection is a major public health problem. About 30% of the world's populations have been infected at some time in their lives with the hepatitis B virus (HBV) (Iarovoi *et al.* 2008). It is estimated that 350 million people have chronic HBV infection and eventually 15% to 25% of

these individuals will progress to liver cirrhosis and hepatocellular carcinoma. About one million die each year from chronic liver disease (Lu *et al.* 2008). More than three-quarters of HBV infections occur in Asia, the Middle East and Africa (Jadallah *et al.* 2005).

HBV is partial double strand circular DNA virus belongs to hepadnaviridae family. It has a unique replication mechanism that replicate via reverse transcription of pregenomic RNA (Oropeza *et al.* 2007). HBV have eight genotypes from A-H. The deference in genotypes affects the severity, complication, course of disease and treatment (Olinger *et al.* 2008).

HBV genotypes have distinct geographical distributions in which multiethnic population tend to have multiple genotypes (Valsamakis, 2008). Genotype A is distributed gobally and is the predominant genotype in North America, North West Europe, India and central Africa (Olinger *et al.* 2008). Genotype B and C are predominant in east and south Asia and Australia (Song *et al.*, 2005). Genotype D has been reported globally but mainly found in middle east, Mediterranean countries and India. Genotype E seems to be predominant in West Africa but genotype F is found in American native, Polynesia, central and South America. Genotype G is found in few patients in France, America, and Germany. Genotype H is found exclusively in central and South America (Mello *et al.*, 2007; Mojiri *et al.*, 2008). Phylogenetic analysis has shown that B/C recombinants have spread through East Asia and that A/D recombinants exist in Italy and C/D hybrid is present in Tibet (Cui *et al.*, 2002).

Methods

Patients

In this study serum samples were taken from 19 suspected patients, 14 males (73.68%) and 5 females (26.32%)> the serumes were provided by Central laboratory in Sulaimani and stored in –20 °C till the DNA was extracted.

DNA extraction

The DNA was extracted from 200 µL serum samples by the QIAamp DNA extraction mini kit (QIAGEN, Germany) According to the manufacturer's instructions.

Detection of HBV

HBV was detected by amplification of pre-S1 through S genes using universal primers, (P1) sense primer, (S1-2) antisense primer, for detection of all HBV genotypes according to described methods by Naito *et al.* (2001). The total reaction mixture was 40 µL. It made up of 27.8µL of DEPC-H₂O, 1X PCR reaction buffer with 15 mM MgCl₂, 250µM concentration of each

dNTP, 10 pMol primers, 1U Red hot Tag polymerase (Thermo scientific, UK) and 5 μ L of extracted DNA.

The thermocycler (Eppendorf, Germany) was programmed to incubate the samples for initial denaturation at 94°C for 5 minutes, followed by 40 cycles consisted of denaturation at 94 °C for 30 Sec, annealing at 55 °C for 1 minutes and elongation at 72 °C for 1.5 minutes. The final elongation was 72 °C for 5 minutes.

Genotyping Method

Genotyping system based on nested PCR, using type specific primers for determination of six genotypes A through F of HBV according to previous method described by Naito *et al.* (2001).

The nested PCR primers were designed on the basis of the conserved nature of the nucleotide sequences in regions of the pre-S1 through S genes. The genotypes can be determined according to differences in the sizes of amplified DNA, in respect of the six HBV genotypes (Table 1).

Two nested PCRs were performed in different mixture for each sample. (mix A) applied for identification of genotypes A, B, C and (mix B) for genotypes D, E, F. 1 μ L aliquot of the first-round PCR product was added to each of mix A and mix B. The nested PCR mixture made of 29.8 μ L of DEPC-H₂O, 1X PCR reaction buffer with 15 mM MgCl₂, 250 μ M of each dNTP, 10pmole of each type specific primers and 1.25U Red hot Tag polymerase.

The nested PCRs were amplified for 40 cycles with the following parameters; preheating at 94°C for 5 minutes, 20 cycles of amplification at 94°C for 30 s, 58°C for 30 s, and 72°C for 40 s, and an additional 20 cycles of 94°C for 20 s, 60°C for 30 s, and 72°C for 40 s, with the final elongation at 72°C for 5 minutes.

PCR products were electrophoresed in a 1.5% agarose gel and stained with ethidium bromide. The PCR bands were then visualized by UV transilluminator. The sizes of PCR products were estimated according to the migration pattern of a 100bp DNA ladder. The genotypes of HBV were determined according to the amplified size of PCR product.

Result

Detection of HBV

A total of 19 suspected hepatitis B patients, 14 male (73.68%) and 5 female (26.32%), were examined by PCR assay using universal primer for detection of all HBV genotypes. 15 patients (78.95%) were negatives and 4 patients (21.05 %) were positives for HBV. The percentage of infected male about (14.29%) and the percentage of infected female about (40%)

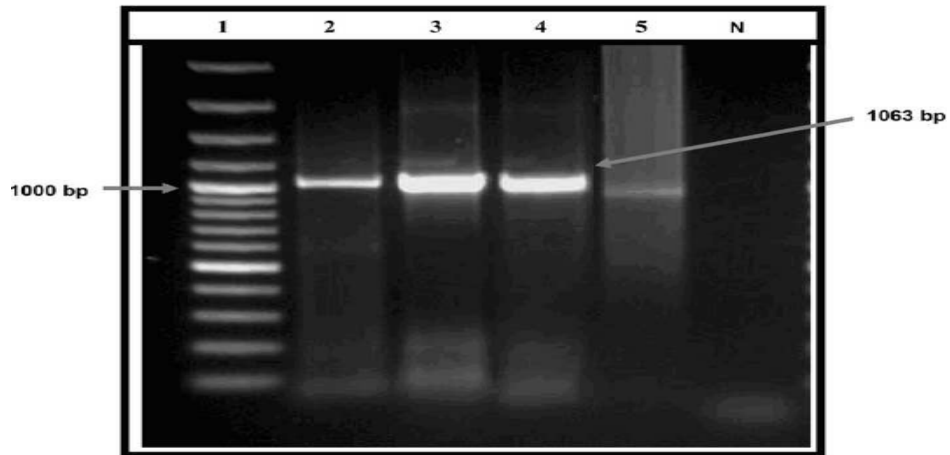


Fig. 1. Positive samples detected by PCR, universal outer primer product (1063bp) used for detection of all HBV genotypes followed by ethidium bromide staining, gel electrophoresis; Lane 1: 100bp DNA ladder, Lane 2-5: positive sample. Lane 6: negative control

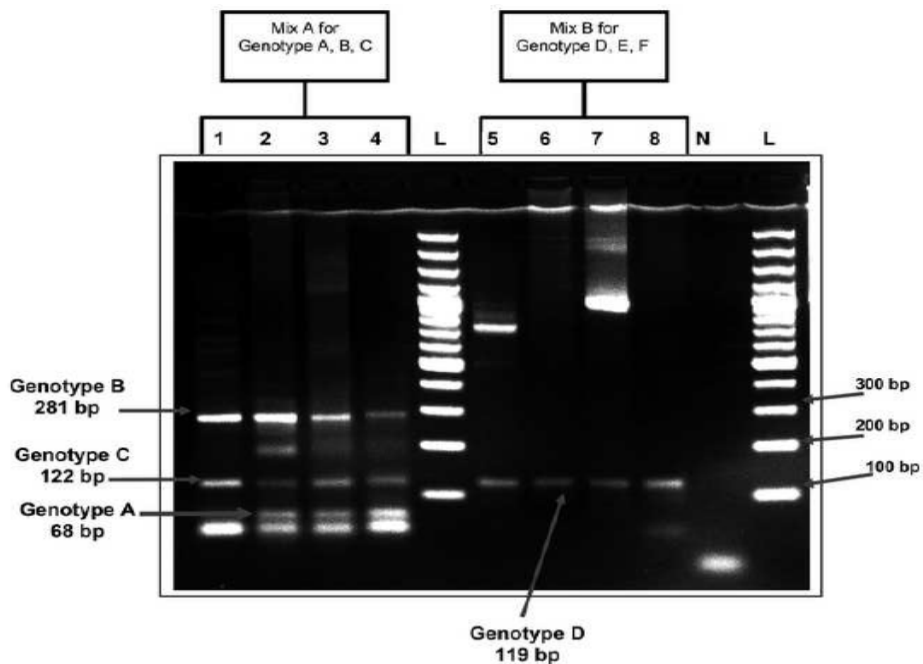


Fig. 2. Genotyping of HBV by type specific genotype based on nested PCR Mix A contain mixture of nested primer for identification of genotypes A, B, and C, lane 1: shows genotypes B and C. lane 2-4: shows genotypes A, B, C. Mix B contain mixture of nested primer for identification of genotypes D,E and F. lane 5-8 show genotype D. N: Negative control. L: represent 100bp DNA ladder

HBV Genotypes Analysis

The 4 positive HBV samples were subjected to genotyping by PCR using type specific primer. 4 genotypes (A, B, C, D) out of 6 genotypes which can be determined by this method were identified. Unusual result were detected in which all of the samples (100%) showed mixed infection and no single genotype was detected in the patients. 1 patient (25%) had mixed genotypes B+C+D and 3 patients (75%) had mixed genotypes of A+B+C+D (Fig. 2).

In order to certify that these bands were not primer dimmer or unspecific band due to present of several primers in the mixture, the specific genotypes primer set were put individually in the PCR mixture for the same sample to perform simplex PCR. The results showed bands of the same expected size for each HBV genotype as in multiplex primer (Fig.3).

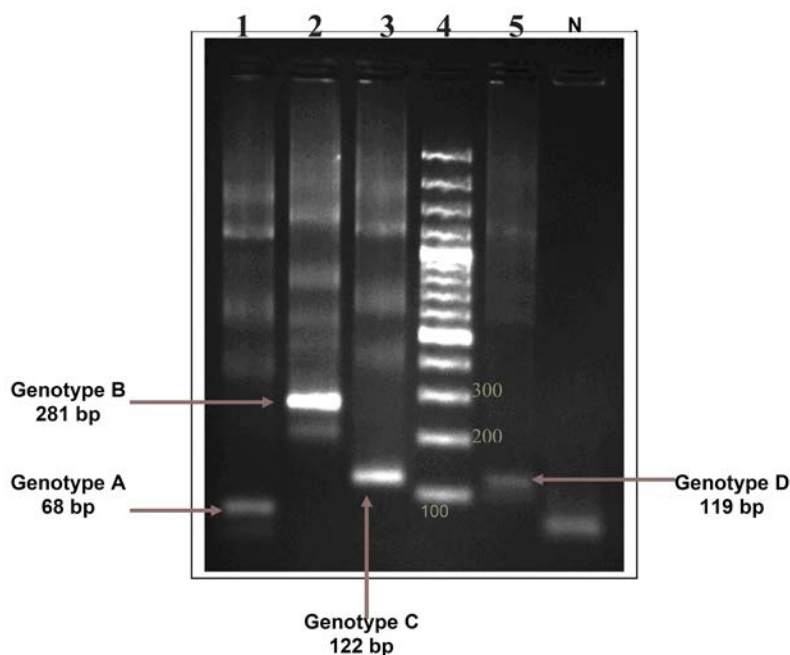


Fig. 3. Genotyping of HBV by PCR using single primer set, specific for HBV genotypes. Lane 1: indicate genotype A 68 bp Lane 2: indicate genotype B, 281 bp. Lane 3: indicate genotype C, 122bp. Lane 4: 100bp DNA ladder. Lane 5: indicate genotype D 119bp. N: negative control.

Table 1. List of primer position, specificity and polarity

First PCR			
P1	universal, sense	5'-TCA CCA TAT TCT TGG GAA CAA GA-3' (2823–2845 nt)	1063bp
S1-2	universal,anti sense	5'-CGA ACC ACT GAA CAA ATG GC-3' (704-685 nt)	
Nested PCR			
Mix A			
Sense			
B2	genotype A to E specific	5'-GGC TCM AGT TCM GGA ACA GT-3' (67– 86, nt,)	
antisense			
BA1R.....	genotype A specific ,	5'-CTC GCG GAG ATT GAC GAG ATG T-3' (134-113 nt,)	68 bp
BB1R	type B specific	5'-CAG GTT GGT GAG TGA CTG GAG A-3' (345-324 nt,)	281 bp
BC1R	type C specific	5'-GGT CCT AGG AAT CCT GAT GTT G-3' (186 -165 nt)	122bp
Mix B			
sense			
BD1	type D specific	5'-GCC AAC AAG GTA GGA GCT-3' (2979–2996 nt,)	119bp
BE1	type E specific	5'-CAC CAG AAA TCC AGA TTG GGA CCA-3' (2955–2978 nt)	167bp
BF1 bb	type F specific	5'-GYT ACG GTC CAG GGT TAC CA-3' (3032–3051 nt)	97 bp
antisense			
B2R	types D to F specific.....	5'-GGA GGC GGA TYT GCT GGC AA-3' (3097-3078 nt)	

sulaimani	HGAESFHQSSGILSRPPVGSSSLQSKHRKSRGLGLSQSQGHLLARRQQGRSWSIRAGIHPTA	60
TURKEY .	HGAESFHQSSGILSRPPVGSSSLQSKHRKSRGLGLSQSQGHLLARRQQGRSWSIRAGIHPTA	240
Egypt	HGAESFHQSSGILSRPPVGSSSLQSKHRKSRGLGLSQSQGHLLARRQQGRSWSIRAGIHPTA	196
Iran	HGAESFHQSSGILSRPPVGSSSLQSKHRKSRGLGLSQSQGHLLARRQQGRSWSIRAGIHPTA	196

sulaimani	RRPFGVPEPSGSGHTTN-----PPNS	80
TURKEY .	RRPFGVPEPSGSGHTTNLASKASCLYPSVPRKAAYPVSTFEKHSSSGHVELHNLPPNS	300
Egypt	RRSFGVPEPSGSGHTTNLANKSASCLYQSPVRKAAYPVSTFEKHTSSSGHVELHLYPPNS	256
Iran	RRPFGVPEPSGSGHTTNLASKASCLHQSPVRKAAYPVSTFEKHSSSGHVELNNIPPNS	256
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sulaimani	ARSQSERPVFPCCWLLQFRNSKPCSDYCLSHIVNLLLEDWGPCAHEGHHIRIPRTPARVTG	140
TURKEY .	ARSQSERPVFPCCWLLQFRNSKPCSDYCLSHIVNLLLEDWGPCAHEGHHIRIPRTPARVTG	360
Egypt	ARSQSERPVFPCCWLLQFRNSKPCSDYCLSHIVNLLLEDWGPCAHEGHHIRIPRTPARVTG	316
Iran	ARSQSERPVFPCCWLLQFRNSKPCSDYCLSHIVNLLLEDWGPCAHEGHHIRIPRTPARVTG	316

sulaimani	GVFLVDKNPHNTAESRLVVDFSQFSRGNRYRVSWPKFAVPNLQSLTNLLSSNLSWLSLDVS	200
TURKEY .	GVFLVDKNPHNTAESRLVVDFSQFSRGNRYRVSWPKFAVPNLQSLTNLLSSNLSWLSLDVS	420
Egypt	GVFLVDKNPHNTAESRLVVDFSQFSRGNRYRVSWPKFAVPNLQSLTNLLSSNLSWLSLDVS	376
Iran	GVFLVDKNPHNTESRLVVDFSQFSRGHHRVSWPKFAVPNLQSLTNLLSSDLWSLDVS	376
*****:*****:*****:*****		
sulaimani	AAFYHLPLHPAAMPPLLVGSSGLSRIVARLSSNSRIFNYQHGTMQNLHDSCSRNLYVSL	260
TURKEY .	AAFYHLPLHPAAMPPLLVGSSGLSRIVARLSSNSRIFNYQHGTMQNLHDSCSRNLYVSL	480
Egypt	AAFYHLPLHPAAMPPLLVGSSGLSRIVARLSSNSRIFNYQHGTMQNLHDSCSRNLYVSL	436
Iran	AAFYHLPLHPAAMPPLLVGSSGLSGYVARLSCNSRIFNYQHGTMQNLHDSCSRNLYVSL	436
***** . *****		
sulaimani	LLYQTFGRKLHLYSHPIILGFRKIPMGVGLSP	292
TURKEY .	LLYQTFGRKLHLYSHPIILGFRKIPMGVGLSP	512
Egypt	LLYQTFGRKLHLYSHPIILGFRKIPMGVGLSP	468
Iran	LLYQTFGRKLHLYSHPIILGFRKIPMGVGLSP	468

Fig. 4. Multiple sequence alignment of polymerase protein by Clastal W2 show deletion mutation of 40 a.a. in Sulaimani isolate.

Sequencing

One sample, lane 5 (Fig. 1), appeared to be smaller in size than the other bands because it migrated faster during electrophoresis. For confirmation the PCR product was sequenced from both ends in IBL laboratory in Vienna. The DNA sequence was published in gene bank as HBV-Sul-01, accession number (GU186046). The amino acid sequence was subjected to multiple sequence alignment by Clastal W2 and showed best identity %87 with polymerase protein of HBV genotype D with large deletion mutation about 40 a.a. with HBV in Iran accession No.AY741798 , Turkey accession No. AAV70943 and Egypt accession No. AB104712 .

Discussion

HBV is characterized by a genetic heterogeneity and 8 genotypes (A to H) can be classified based on comparison of complete HBV genomes (Palumbo et al., 2008). The genotyping of HBV is important to clarify the route and pathogenesis of the virus because HBV variants may differ in their patterns of serologic reactivity, pathogenicity, virulence, and response to therapy and global distribution (Naito et al., 2001; Baig et al., 2007)

Several methods have been developed and used for HBV genotyping including direct sequencing, PCR based restriction fragment length polymorphism, line probe assay, enzyme-linked immunoassay (Zekri et al., 2007). Reverse hybridization, real-time PCR with melting curve analysis and oligonucleotide chips. However, the sensitivity, specificity, cost, and time required are different in these methods (Wang et al., 2007). New HBV genotyping method established based on PCR amplification assay using type specific primers (TSP), which can identify six major genotypes. In designing the genotype-specific PCR primers, it is well established that not only higher matching in the entire sequences but also the matching of the two to three nucleotides at the 3' ends is one of the important parameters for specific priming (Naito et al., 2001). In comparison with other methods of genotyping such as RFLP, this method seems to have a higher sensitivity of detection and higher capacity in distinguishing HBV genotypes. The sensitivity of detection using this method was 102 copies/ml (Moriyama et al., 2003; Geramizadeh et al., 2008)

For these reasons, the TSP-PCR was employed for HBV genotyping in this study to investigate the prevalence of HBV genotypes in Sulaimani, Kurdistan region of Iraq among Kurdish people, which was the first study done for molecular detection and genotyping of HBV by PCR. The result indicated that out of 19 patients 4 patients were positive for HBV. the 4 positive samples subjected to genotyping by TSP-PCR. Unique results were detected, that no single genotype were identified and all of the samples showed mix infection of A B C D.

Reports about HBV genotypes in the neighboring countries illustrated that; In Iran, study among 80, 89 and 60 positive cases show 100% prevalence of genotype D (Vaezjalali et al., 2002; Geramizadeh et al., 2008 Mojiri et al., 2008). In Turkey Genotype D represent almost the whole Turkish patients population infected with HBV (Bozdayi et al., 2008). In Saudi Arabia a research which include 70 patients, (81.4%) of them had genotype D, (1.4%) had genotype A, (1.4%) had genotype C, (5.7%) had genotype E, while 7 patients (10%) had mixed genotypes (4 patients A+D+G, 1 patient D+E, 1 patient D+F, and 1 patient A+D+F+G) (Abdo Ayman et al., 2006). In Pakistan, genotyping by this method among 295 positive cases indicated genotype D, A, and (9.1%) showed mix infection by A+B (Baig et al., 2007). In Egypt, genotyping by this method among 70 patients indicated infections by single genotype A, B, C, D, and 11 cases showed mixed genotypes. Five cases had both genotypes A+D, 2 cases had C+D, 2 other cases had B + D and 2 cases had genotypes B+C (Zekri et al., 2007).

The high prevalence of mixed infections in this study may be due to the migration of a large number of Kurdish people as refugee to Europe and America and remaining in the refugee camps for long periods, in which communication with people of different countries might cause transmission of infection with different genotypes. Another factor might be low level of education about the way of disease transmission in Kurdistan (Samir et al., 2013). Negligence of sterilization in the dental clinics and negligence of using disposable materials make up and hairdresser salons may be another factor for coinfection with different HBV genotypes.

The effect of the large deletion mutations were not known, further research is needed to determine the effect of this mutations on clinical disease of the patient and the patients must follow up to determine the effect of mix infections by 4 genotypes related to the course of the disease, HBeAg seroconversion and response to treatment.

Acknowledgments

This work was carried out with financial support from Kurdistan Institute for strategic studies and scientific researches, University of Sulaimani. Kind regards to Prof. Aumaid Uthman for his helps in submitting the sequence in NCBI gene bank.

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